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TITLE: Use of Intraductal Adenovins Transduction to Assess the Mammary Tumorigenic Potential of a Constitutively Active Prolactin Receptor

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We have made good progress in establishing adenovirus transduction as a method for the short term alteration of gene expression in the mammary epithelium. Thus, detailed conditions for reasonably efficient transduction of the mammary epithelium of the late pregnant mouse have been worked out, transduction with adenovirus and expression of green fluorescent protein, GFP, have been shown not to interfere with the function of the mammary cells in which they are expressed. Transgenic technology has been used to evaluate the effects of an activated prolactin receptor, aPRLR, and an activated member of the prolactin signal transduction pathway, Akt, on the mammary epithelium. The latter has been shown to prolong mammary involution and preliminary results suggest it may enhance tumorigenesis. Some changes in direction are warranted: A functional proof of principle experiment utilizing expression of the cytoplasmic tail of the tight junction protein, occludin, is in progress. Adenovirus expressing Akt will be used with a mouse model of tumorigenesis, a mouse overexpressing the *neu* oncogene, to examine the question of whether adenovirus can be used to express genes that promote tumorigenesis.

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Abbreviations used: GFP, green fluorescent protein; WGA, wheat germ agglutinin; pfu, plaque-forming units; Cy3, a red fluorescent used for visualization of cell structures in the presence of GFP; DAPI, a blue nuclear stain; aPRL-R, activated prolactin receptor; Akt, a signal transduction intermediate in the PRL signalling pathway, also known as PKB;

INTRODUCTION

The stated objectives of the proposed research are to

1. Explore the use of adenovirus vectors for localized transduction of the mammary epithelium *in vivo* with genes of known tumorigenic potential.

2. Utilize a constituatively active prolactin receptor to test the hypothesis that long-term stimulation of the prolactin receptor pathway promotes mammary tumorigenesis.

3. Use antibodies specific for signal transduction intermediates to investigate the state of signaling molecules in specific cells.

We have spent the majority of time so far on the first objective and have derived some alternative methods for determining whether adenoviral transduction of the *in vivo* mammary epithelium is a viable method of modifying mammary tumorigenesis in mice. The second objective has been achieved in part using transgenic mouse technology. We have begun exploration of the third objective.

BODY

Task 1: Construction of Adenovirus vectors.

Adenovirus vectors have been constructed with lacZ and green fluorescent protein (GFP) cDNAs. In addition we have constructed an adenovirus vector with the cytoplasmic tail of the tight junction protein occludin. This construct was not included in the original statement of work but has been constructed for the purpose stated under task 2.

Task 2: Studies of efficacy and persistence of adenovirus vectors in various reproductive stages.

A. *Improvement of transduction methodology*

In last year's report we stated that transduction of up to 20% of the epithelial cells in the mammary gland had been achieved with intraductal injection of adenovirus bearing the gene for a marker protein, LacZ. In attempting to repeat these experiments we obtained a much lower efficiency of transduction and therefore increased the dose of virus from about 10^7 pfu (plaque forming units) to doses up to 10^{10} pfu. We examined:

a. The histology of the gland

b. The degree of transduction by staining with the LacZ substrate

c. The state of the tight junctions using a test derived in this laboratory and described in the original proposal: ^{14}C-sucrose is injected into the mammary duct through the nipple canal and its appearance in the blood stream monitored. During lactation , there should be no appearance of the tracer in the blood, because the tight junctions between epithelial cells are tightly closed.

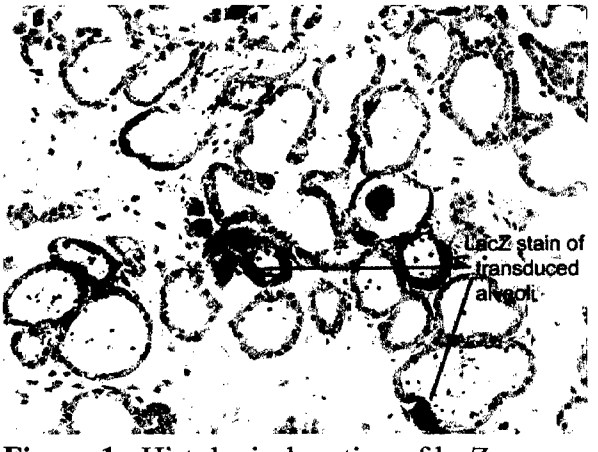

Figure 1. Histological section of lacZ transduced mammary gland. Note stained cells in extended interstitial space, a sign of inflammation..

Our findings:

a. When 10^8 pfu virus and above were injected into the 4th mammary gland, a severe inflammatory could be observed histologically, the transduction efficiency was not markedly improved, and the tight junctions were open.

b. With 10^7 pfu using lacZ as a marker the junctions were closed but the gland showed patchy transduction and some signs of inflammation (Figure 1).

c. Using 10^7 pfu of virus containg the cDNA for GFP as a marker and diluting the virus to the highest volume that could be injected into the 4th mammary gland (about 200 µl) we obtained no inflammation and highly efficient transduction (Figure 2). Preliminary estimates suggest that about 50% of alveoli were transduced and at least 50% of the cells in these alveoli were transduced with the green fluorescent protein. Quantitation of these results is in progress.

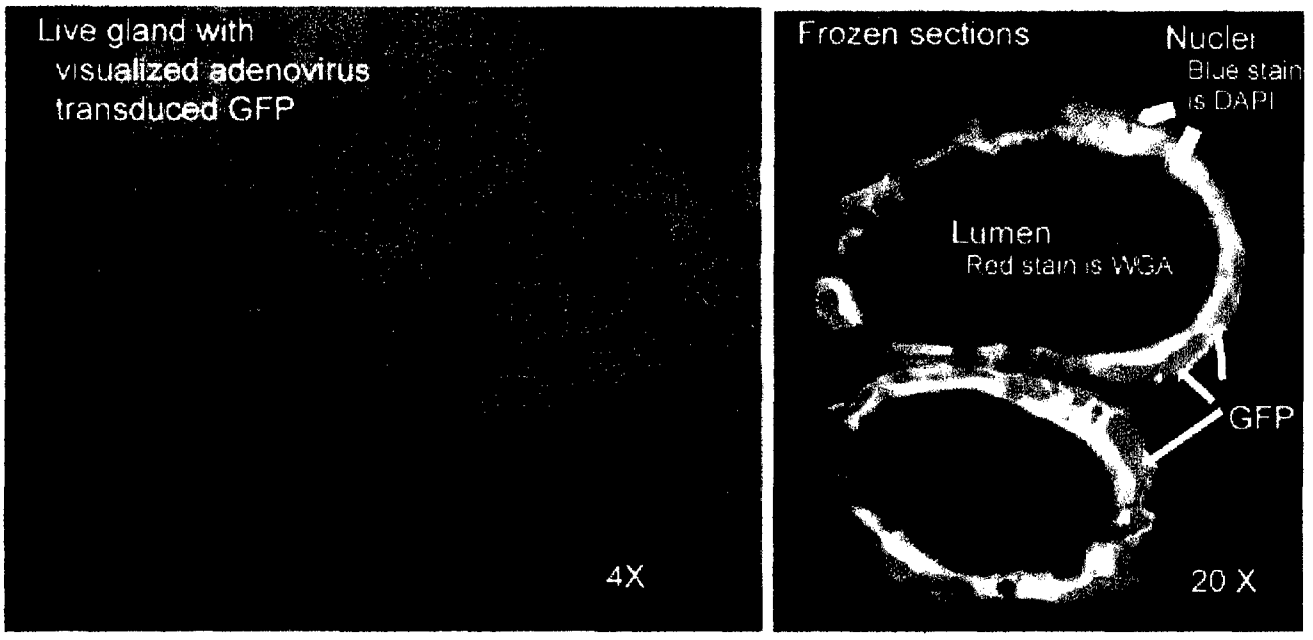

Figure 2. Images from 4th mammary gland transduced with adenovirus containing the GFP (green fluorescent protein) gene at late pregnancy and evaluated during lactation. Left: Gland visualized in the live animal under the dissecting microscope. Note extensive alveolar stainging. Right: Sections of transduced gland stained with Cy3-labeled wheat germ agglutinin for apical mucins (WGA) and DAPI for nuclei.

B. *Effect of reproductive phase on adenoviral transduction.*

We have found that efficient transduction of alveoli occurs in late pregnancy but not in lactation, presumably because milk products interfere with viral binding to the epithelium. We have therefore focused our efforts on the following protocol: virus is injected on day 18 of pregnancy and the mice are examined for extent of transduction, inflammation and other functional parameters on day 1-2 of lactation, 3 days later. With this time course we achieve no inflammation and a high degree of transduction. In the future we will examine transduction of the virgin gland.

C. *Effect of optimal adenoviral transduction on physiological function*

Using the experimental protocol in B we have examined tight junction closure using the ^{14}C-sucrose protocol and morphological assessment of milk secretion by cells that are virally transduced. In these animals there was no passage of ^{14}C-sucrose into the blood stream, indicating that the junctions close normally during the transition from pregnancy to lactation in transduced cells. In addition milk fat droplets appeared to be secreted normally judging from the immunocytochemical localization of the milk fat globule protein, xanthine oxidase and the presence of casein in both cells and alveolar lumina of transduced cells. Therefore transduction with virus coding for GFP does not alter mammary cell function. **[N.B. This essential functional test was not in the original statement of work]**

D. *Can adenoviral transduction of a dominant negative gene alter mammary function?*

Because it appeared from studies in transgenic mice that a constitutively active prolactin receptor had little or no effect on mammary tumorigenesis (see below), we decided that an experimental *proof of principle* was essential at this junction. In essence it is necessary to show that adenoviral transduction can alter physiological function *in vivo*. Because we are very familiar with the test for tight junction integrity described above and because a change in tight junction permeability is an accompaniment of the transition from pregnancy to lactation, we took the following approach which is currently in progress:

We have made an adenoviral construct containing the cytoplasmic tail of the tight junction protein occludin. Using a retroviral system this gene product has previously been shown to interfere with tight junction closure in tissue culture systems (1). We hypothesize that expression of this gene will interfere with tight junction closure in the *in vivo* system. A Flag-tagged adenoviral construct design to express both the cytoplasmic tail of occludin and GFP has been made and is available in large quantities. We have done one preliminary experiment in which we found that the degree of closure of tight junctions in glands transduced with the occludin cytoplasmic tail was less than that observed in control glands transduced with GFP alone. The results of this experiment, which requires repetition, suggest that the adenoviral system can, indeed, be utilized to express functionally active genes in the *in vivo* mammary epithelium. These experiments are in progress at the writing of this report so additional information is not available at this time. **[This essential proof of principle experiment was not part of the original statement of work. Considerable time has been devoted to it because it is essential to show short-term efficacy of adenoviral transduction prior to embarking on the longer term experiments necessary to show effects on tumorigenesis].**

Experiments remaining in task 2:

Completion of proof of principle experiment with occludin tail.

Determination of virus persistence using the improved injection protocol.

Examination of transduction efficiency and persistence in the virgin gland.

Task 3. Transduction with known oncogenes

To be done in the future if it the experiment described under task 6 is positive.

Task 4. Transduction with activated prolactin receptor (aPRLR)

Because the adenovirus technique appeared to require more work before it could be reliably used to examine tumorigenesis by any agent, we made transgenic mice using the gene for the activated prolactin receptor (aPRL-R) on a mammary specific promoter. We were able to obtain expression of the gene in the mammary epithelium but to date (about 1 year after founders were obtained) we have seen no tumorigenesis. This fact suggests to us that use of adenovirus for this purpose is not a good experiment. We therefore consider this task completed to have been completed using a transgenic approach.

Task 5. Transduction with activated JAK2 and STAT5.

We have used a different member of the prolactin signal transduction pathway, the intermediate kinase, Akt (also known as protein kinase B or PKB). We have utilized a constitutively active form of this molecule to make transgenic mice utilizing the mammary specific promoter, MMTV. These mice have been extensively analyzed and show defects in alveolar development, a reduction in milk production in lactation, and very delayed involution (K Stack and S. Anderson, manuscript in preparation). A few mice have been subjected to multiple pregnancies and two have so far developed tumors. These experiments suggest that the prolactin receptor pathway is involved in tumorigenesis. These results also suggest that, if persistence of the adenovirus after mammary involution can be acheived, the interaction of Akt with a known tumorigenic gene will be a worthwhile test of the utility of adenovirus for studies of tumorigenesis. The appropriate experiment is outlined below under Task 6.

Task 6. Evaluation of tumorigenesis

Tumorigenesis has been evaluated in transgenic mice using using aPRL-R and activated Akt as described above. In preparation for tests of the utility of adenovirus for studies of tumorigenesis we are evaluating the latency of tumor development in mice that overexpress the *neu* oncogene. In these mice, obtained from the Jackson laboratory, we have found that mammary tumors begin to appear at six months by nine months 50% of the mice have tumors (Figure 3). This model will be used to evaluate adenovirus transduction as a tool in tumorigenesis research assuming that we observe that expression of adenovirus lasts at least three weeks after involution. Mice overexpressing the *neu* oncogene will be made pregnant at seven months of age. Adenovirus expressing activated Akt and GFP will be injected into the left fourth mammary gland at day 18 of pregnancy. As a control adenovirus expressing activated GFP will be injected into the right 4th mammary gland. The pregnancy will be allowed to proceed as usual and the mice will be examined for tumor development until they are 14 months of age. The finding that a higher proportion of tumors develop in the left mammary gland during this period than in the right gland will be taken as evidence that adenoviral-expressed genes can be used to evaluate tumorigenesis. We anticipate that 25 mice will be

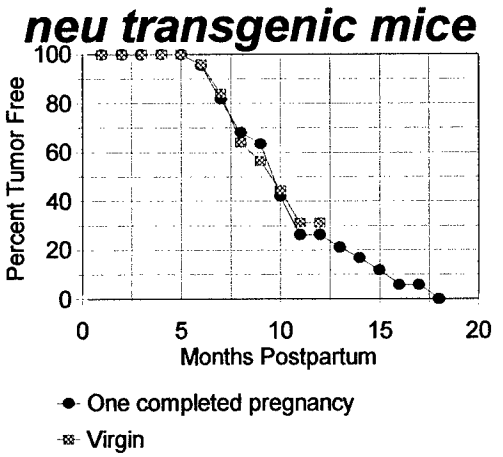

Figure 3. Tumor latency in neu transgenic mice. Mice were either bred at 8 weeks of age and allowed to nurse one litter or they were maintained as virgins. N in each group = 25.

needed for this experiment. Another six mice will be injected and sacrificed at day 6 after involution to examine persistence of transduction and effects on the time course of involution.

Task 7. Evaluations of alterations of signal transduction pathways.

This task is in progress using the transgenic mice overexpressing activated Akt under the MMTV promoter. In particular cellular localization of both total and phosphorylated Akt is being visualized. Both forms of the protein can be seen in cells, however, detailed analysis of expression has only begun at this point.

(6) KEY RESEARCH ACCOMPLISHMENTS

Our key accomplishments to date are:

- Construction of viruses suitable for expression of GFP, Lac Z and the cytoplasmic tail of occludin. (A complete listing of viruses was presented in the previous report)
- Putting intraductal injection in the mouse on a firm technical basis, with a technique that can be described and taught to others (see paper in press, below)
- Achieving reproducible expression of GFP in a high proportion of mammary cells and alveoli under conditions where no inflammation can be detected in the gland 3 days post-transduction.
- Evaluation of the functional competency of transduced mammary epithelium
- Use of transgenic technology to show that overexpression of an activated PRL-R in the mammary epithelium does not lead to mammary tumorigenesis
- Use of transgenic technology to show that overexpression of Akt, a member of the prolactin signal transduction pathway, has effects on the mammary gland which include alterations in milk secretion, a delay in mammary involution following lactation and, potentially, enhanced mammary tumorigenesis.

(7) REPORTABLE OUTCOMES

During the period 15-Sept-99 - 14-Sept-00 we have mostly been at work attempting to make this technique reproducible and examining the functional consequences of expression of adenovirus mediated genes in the mammary epithelium. However, we have presented two Abstracts during this time period:

Injection of adenovirus vectors in the mouse mammary gland achieves efficient, long term transduction of mammary epithelial cells without inflammation. Neal Beeman and Margaret C. Neville. American Society for Cell Biology, December 1999.

Transduction of Epithelial Cells by intraductal injection of adenovirus vectors into the mouse mammary gland. Margaret C. Neville and Neal Beeman. Era of Hope Meeting, June 2000.

One paper is still in press:

Intraductal injection into the mouse mammary gland. 2000. Duy-Ai Nguyen, Neal Beeman, Michael Lewis, Jerome Schaack, Margaret C. Neville. In M.M. Ip and B.B. Asch, *Eds, Research*

Methods in Mammary Gland Biology and Breast Cancer. Kluwer Academic/Plenum Publishers. In Press.

(8) CONCLUSIONS

We have made good progress in establishing adenovirus transduction as a method for the short term alteration of gene expression in the mammary epithelium. Thus, detailed conditions for reasonably efficient transduction of the mammary epithelium of the late pregnant mouse have been worked out, transduction with adenovirus and expression of green fluorescent protein, GFP, have been shown not to interfere with the function of the mammary cells in which they are expressed. Transgenic technology has been used to evaluate the effects of an activated prolactin receptor, aPRLR, and an activated member of the prolactin signal transduction pathway, Akt, on the mammary epithelium. The latter has been shown to prolong mammary involution and preliminary results suggest it may enhance tumorigenesis. Some changes in direction are warranted: A functional proof of principle experiment utilizing expression of the cytoplasmic tail of the tight junction protein, occludin, is in progress. Adenovirus expressing Akt will be used with a mouse model of tumorigenesis, a mouse overexpressing the *neu* oncogene, to examine the question of whether adenovirus can be used to express genes that promote tumorigenesis.

(9) REFERENCE

1. Bamforth SD, Kniesel U, Wolburg H, Engelhardt B, Risau W J A dominant mutant of occludin disrupts tight junction structure and function. 1999 Jun;112 (Pt 12):1879-88.

(10) APPENDICES

Copies of the two abstracts mentioned above are included as appendices. A preprint of the article was sent previously.

Injection of adenovirus vectors into the mouse mammary gland achieves efficient, long term transduction of mammary epithelial cells without inflammation. Neal Beeman and Margaret C. Neville. American Society for Cell Biology, December, 1999.

A non-invasive, non-inflammatory, gene delivery system is needed for controlled temporal and spatial expression of introduced genes in the mammary epithelium. Adenovirus vectors encoding either LacZ or GFP reporter genes were injected into the mammary lumen through the teat canal. LacZ transduced glands were processed for histochemistry. GFP transduced glands were surgically reflected, maintaining blood flow, and observed in living mice. Transduction was patchy and much of the gland was negative; however, up to 100% of cells were positive for reporter activity in transduced areas. Only luminal cells were transduced. Lactation appeared to be unaffected. Transduced alveoli were distended with milk and milk fat globules. Milk fat globules were observed within transduced cells and immunofluorescence demonstrated the presence of milk proteins in morphologically normal transduced cells. Transduced glands did not show lymphocytic invasion and transduction persisted for over 60 days without pathology. A population of transduced cells has been shown to survive the normal involution of the mammary gland at the end of lactation. These findings suggest that adenovirus vectors provide an effective means to alter the genetics of the mammary epithelium in a spatially and temporarily controlled manner.

Supported by DOD Grant BC971759.

TRANSDUCTION OF EPITHELIAL CELLS BY INTRADUCTAL INJECTION OF ADENOVIRUS VECTORS INTO THE MOUSE MAMMARY GLAND

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A non-invasive, non-inflammatory, gene delivery system is needed for controlled temporal and spatial expression of introduced genes in the mammary epithelium. Adenovirus vectors endoding either LacZ or GFP reporter genes were injected into the lumen of the fourth mammary gland through the teat canal. LacZ transduced glands were processed for histochemistry. GFP transduced glands were surgically reflected attached to the skin, maintaining blood flow, and observed in living mice. Severe and destructive inflammation of the gland was observed when 10^8 or 10^{10} plaque forming units (pfu) were injected into the gland. Minimal inflammation was observed 3 days after introduction of 10^7 pfu into the 4th gland of 17 day pregnant mice. Transduction of the epithelium was maintained through the transition to lactation at parturition. Only cells of the mammary epithelium were transduced. Up to 100% of cells in certain alveoli were transduced, however, transduction was patchy and much of the gland was negative. In transduced alveoli lactation appeared to be unaffected. Transduced alveoli were distended with milk and milk fat globules. Milk fat globules were observed within transduced cells and examination by immunofluorescence demonstrated the presence of milk proteins in morphologically normal transduced cells. These findings suggest that adenovirus vectors provide an effective means to alter the genetics of the mammary epithelium in a spatially and temporarily controlled manner. However, uniform transduction of the epithelium with low doses of vector has not yet been achieved.

The U.S. Army Medical Research and Materiel Command under DAMD17 98-1-8296 supported this work.